Nucleotide-dependent conformations of the kinesin dimer interacting with microtubules

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Background: Kinesins are crucial to eukaryotic cells. They are a superfamily of motor proteins that use ATP hydrolysis to move along microtubules. Many of these motors are heterotetramers with two heavy and two light chains. The heavy chain has a globular motor domain that interacts with microtubules and shows a similar sequence throughout the family. Compared with myosin and dynein, kinesin provides a 'simple' model for understanding molecular motors.

Results: Electron cryomicroscopy and three-dimensional reconstruction methods have been used to investigate microtubule–kinesin dimer complexes in different nucleotide states. Three-dimensional maps were obtained in the presence of 5′-adenylylimidodiphosphate (AMP–PNP), ADP–AlF_4, ADP and apyrase. In all cases, kinesin has one attached and one free head per tubulin heterodimer. The attached heads appear very similar whereas the free heads show distinct conformations and orientations depending on their nucleotide states.

Conclusions: The kinesin dimer is likely to undergo considerable conformational changes during its ATP hydrolysis cycle. In all nucleotide states, the kinesin dimer attaches to a microtubule using one motor domain with the other motor domain hanging free. Only the free domain changes conformation in the presence of different nucleotides, suggesting that it, or the region linking both motor domains to the coiled coil, is the determinant of directionality. These results give some structural clues as to how kinesin moves along microtubules and we describe possible models of kinesin movement based on currently available data.

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Introduction

The kinesin family of motor proteins, in partnership with microtubules, are involved in many important processes in eukaryotic cells, such as cell division, intracellular transport and organisation of the cytoplasm [1]. Most of these motors are heterotetramers with two heavy and two light chains. Each heavy chain has a globular region, the motor or catalytic domain, that binds ATP, interacts with microtubules and shows a high degree of sequence homology throughout the family. The light chains, together with the distal part of the heavy chain are thought to determine the specificity of the cargo transported along microtubules. Kinesin itself is a processive motor; it can travel considerable distances along microtubules without falling off, and it moves with a step length of ~8 nm along the protofilament direction towards the microtubule plus end [2–4]. Some other members of the family, such as ncd (*Drosophila* non-claret disjunctional protein) move in the opposite direction [5]. Three-dimensional maps of microtubules complexed with kinesin and ncd dimers in the presence of 5′-adenylylimidodiphosphate (AMP–PNP) have previously been obtained by cryomicroscopy and helical reconstruction methods [6,7]. In these structures, the free heads of kinesin and ncd pointed towards their respective directions of movement suggesting that the opposite directionality of the motors might be related to the free-head conformations. Apart from this, and despite the existence of X-ray crystal structures for kinesin and ncd monomers [8,9], there is still a lack of structural data to guide our understanding of how kinesin motors move along microtubules.

Microtubules are the cytoskeletal pathway used by the motor proteins. They are hollow cylinders with a diameter of ~25 nm and are built from heterodimeric tubulin. The dimers are aligned head-to-tail along protofilaments that associate laterally with a slight offset to form the microtubule wall. This arrangement confers a structural polarity to microtubules and at the present time it is believed that the β subunit is located at the plus end [10–12]. Several experiments have been described for the determination of this structural polarity. The best method for the three-dimensional reconstruction work described here is to determine the polarity directly by using arrowhead moire patterns shown by vitreous-ice embedded microtubules observed by electron cryomicroscopy [13]. Another important structural parameter is the surface lattice. The B lattice is now widely accepted, at least for

in vitro assembled microtubules [14]. In this kind of organisation, the shallow pitch helices in the microtubule wall are formed by alternate rows of α or β subunits. If the number of helices at the tubulin monomer level is even, then the microtubule is strictly helical; if the number is odd, there is a discontinuity in the surface lattice. To apply standard helical reconstruction methods, we introduced the use of microtubules with 15 protofilaments and four shallow-pitch helices (i.e. four-start monomer helices; 15:4 microtubules) that occur as a minor component during *in vitro* microtubule assembly [6].

The aim of this work was to determine the different conformational steps involved in the movement of kinesin along microtubules. We chose kinesin as a model because, at the present time, this is the best characterised processive microtubule motor. We have used recombinant dimeric *Drosophila* kinesin and fully helical microtubules to study the nucleotide-dependent conformations of microtubule–kinesin complexes. Electron cryomicroscopy and three-dimensional reconstruction methods give the structure of microtubules decorated with kinesin dimers in the AMP–PNP (ATP analogue), ADP–AlF_4 (ADP–P_i analogue), ADP and no-nucleotide states. Binding assays have previously shown that kinesin is strongly attached to microtubules in all but the ADP state [15].

Results and discussion

Working conditions

We used the truncated amino-terminal region of *Drosophila* kinesin (called KHC392) that includes amino acid residues 1–392 and extends beyond the minimal motor domain by some 50 amino acids [16]. This recombinant protein expressed in *Escherichia coli* spontaneously forms dimers that are viable motors [16]. To apply standard helical reconstruction methods, we worked with 15:4 microtubules, assembled and stabilised *in vitro* in the presence of taxotere. These fully helical microtubules were complexed with kinesin dimers in the presence of adenosine nucleotides or nucleotide analogues and imaged in vitreous ice using electron cryomicroscopy. In electron micrographs, 15:4 microtubules can be distinguished from other types of microtubule by their characteristic moire patterns and by their computed diffraction patterns [6]. The polarity of individual microtubules was determined from the arrowhead moire patterns visible on microtubules in vitreous ice [6,13] and was confirmed by the protofilament skew in end-on-views of the three-dimensional reconstructions [17].

Microtubule–kinesin complexes in different nucleotide states

Three-dimensional maps of microtubule–kinesin dimer complexes in three nucleotide states (AMP–PNP, ADP and no nucleotide) are shown in Figure 1 as isodensity surfaces, with the plus end of the microtubule oriented upwards. The results with ADP–AlF_4 are not shown because they are essentially identical to the structure of the complex in the presence of AMP–PNP.

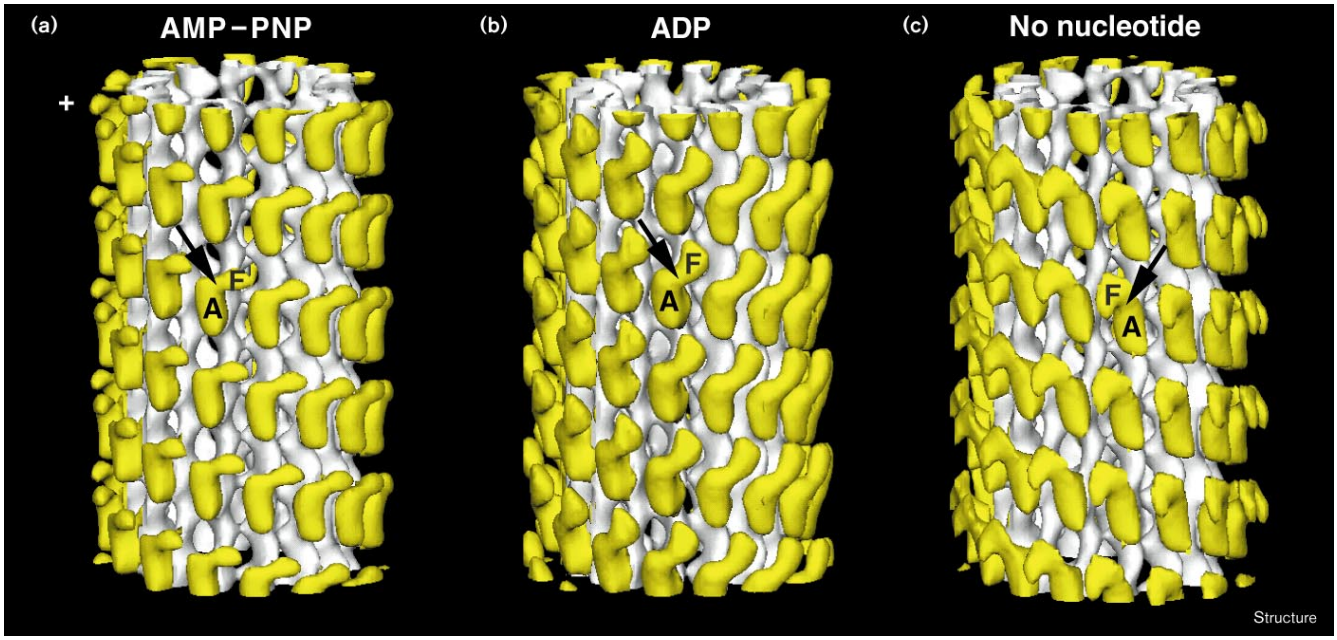
There are several notable features of the structures shown in Figure 1. First, the motor proteins bind specifically every 8 nm along the protofilaments, one motor dimer per tubulin heterodimer. The motors align laterally along the left-handed four-start helix direction showing that inter-protofilament nearest neighbours are identical tubulin subunits, the so-called B lattice [14,18]. In all cases, the motor dimers have one attached head and one free head per tubulin heterodimer. At the resolution of the maps (~35 Å), the attached heads appear very similar for all nucleotide states. Viewed from the microtubule plus end they are attached on the outer crest of the protofilaments with a slightly clockwise skew. Unlike usual representations, heads never stand fully upright. Head A lies flat and stretches from the plus end of one tubulin subunit over the next subunit towards the microtubule plus end. Consequently, head A is close to both the α and β tubulin subunits and probably interacts with them both, as implied by the results of most chemical crosslinking experiments and electron microscopy [6,7,14,19–22]. At its plus end the attached head contacts the free head.

The free heads have distinct conformations and orientations depending on the nucleotide state. In the presence of ADP (Figure 1b), the free head points upwards and to the right of the attached head. The angle between the long axes of the attached and the free head increases from ~110° for AMP–PNP (Figure 1a) and ADP–AlF_4 to ~120° for ADP (Figure 1b). The neck region, indicated by an arrow, is located on the top right of the attached head. In the presence of apyrase (no attached nucleotide), the free head is very different: it is located on the left side of the attached head and slightly curved away from the microtubule. The neck region is now located at the top left of the attached head (Figure 1c). For all nucleotide states, the free head appears to have a smaller volume than the attached head. Sucrose-gradient ultracentrifugation, gel filtration chromatography [16] and crosslinking experiments in our laboratory (data not shown) indicate that these molecules are indeed dimers. The smaller size of the free head in the reconstructions is most probably due to a high degree of flexibility in the neck region leading to conformational disorder that reduces its apparent volume [6,7].

Conclusions

The three-dimensional maps of the microtubule–kinesin dimer complexes in Figure 1 can be thought of as still pictures of kinesin in given nucleotide states. They are highly significant in that we always observe a stoichiometry of one kinesin dimer per tubulin heterodimer with a single kinesin motor domain attached to the crest of a protofilament and skewed slightly clockwise when viewed from the microtubule plus end. The other motor domain is

Figure 1



Three-dimensional maps of kinesin dimers (yellow) interacting with microtubules (white) in the presence of **(a)** 5′-adenylylimidodiphosphate (AMP–PNP), **(b)** ADP, and **(c)** apyrase (no nucleotide). As discussed in the text, kinesin shows distinct, nucleotide-dependent conformations. The plus end of the microtubules is oriented upward. The arrows indicate the region between the attached (A) and the free (F) heads that probably corresponds to part of the 'neck'.

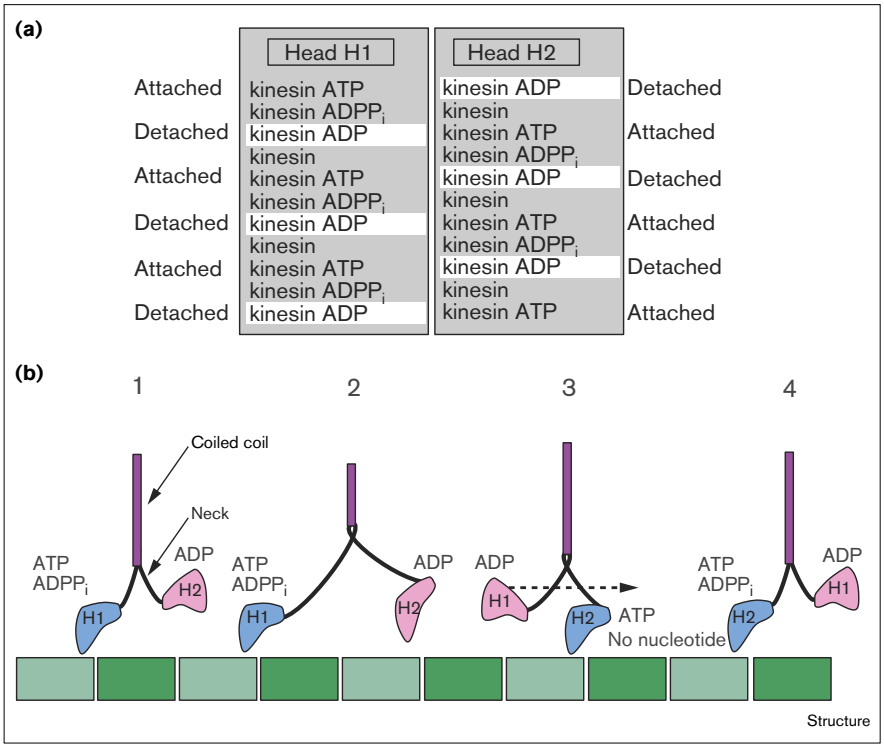
unattached. The attached monomer is seen to have similar positions and conformations for all four conditions used to prepare microtubule–kinesin complexes: AMP–PNP, to imitate the ATP state; ADP–AlF_4 to imitate ADP–P_i; ADP; and apyrase to imitate the no-nucleotide state. The AMP–PNP, ADP–AlF_4 and no-nucleotide states are known to be strongly bound [15]. Kinesin in the ADP state is known to bind weakly to microtubules, but there is conflicting evidence concerning the subsequent release of ADP [15,23]. Under our working conditions the attached head is likely to be in the ADP state.

In comparison, under the same conditions the conformation of the unattached monomer is seen to be strongly influenced by the nucleotide state. This suggests that the unattached motor monomer plays a major role in the movement and directionality of kinesin. Of course, subtle modifications in the attached head, invisible at the resolution of our maps, could influence the free head via the neck or coiled-coil regions.

The junction between the two motor-domain monomers is at the end of the attached monomer directed towards the microtubule plus end (Figure 1). The attached monomer stretches along a protofilament and partly covers both the α and β tubulin subunits. This is compatible with crosslinking results using a zero-length crosslinker [19,21] and with results of a blot-overlay assay [20] that show kinesin to interact with both α tubulin and β tubulin. In Figure 1, the free monomer points sideways and upwards towards the microtubule plus end. It appears to have an in-built flexibility that reduces its apparent volume in the averaging process leading to the three-dimensional reconstructions. It has sufficient extension to reach one complete dimer distance upwards from the attached monomer, either on the same or a neighbouring protofilament. We have verified this by superposing the full volume of the monomer onto the position of the free monomer. Overall, the kinesin dimer appears to be positioned to move towards the microtubule plus end. Out of the many hypothetical stepping patterns [18,24–26], we suggest that only two are compatible with previous results showing kinesin to move along the protofilament direction [3], in ~8 nm steps [4], and the results presented here. The two compatible patterns are: kinesin takes 8 nm steps along a single protofilament, and kinesin steps along two neighbouring protofilaments with alternate 7 nm and 9 nm steps.

Whatever the detailed molecular mechanisms involved, we expect processive movement along a microtubule to occur by repeating a basic stepping cycle many times. There are a good number of indications that the stepping cycle involves a 'hand-over-hand' process in which one or the other of the motor domains is always in contact with the microtubule [23,27,28]. Consideration of the dimer structures that we observe, and in particular the kinesin–ADP

Figure 2



Schematic model for kinesin movement. **(a)** Coordination of the ATP hydrolysis cycles of the two heads of the kinesin motor protein. Possible synchronisation of the ATP hydrolysis cycle of kinesin's two heads as a function of their attached and free states. **(b)** Possible schematic model of kinesin movement based on currently available data. The heads of the motor protein (H1 and H2) are coloured blue when attached to the microtubule or pink when unattached. The tubulin heterodimer along a single protofilament is represented by the light and dark green rectangles. The mechanism described here could function either along one or two protofilaments. Stage 1: the attached head H1 (ATP or $ADPP_i$ state) is fixed in a 'stable' position on the protofilament and the unattached head H2 (ADP state), connected to H1 by the flexible neck, can search for its future binding site, perhaps through a thermally activated process involving random rotations around its 'tether'. Stage 2: this step corresponds to the hypothetical rotation of H2 to be in the correct orientation to interact with the next binding site along the protofilament direction. Destabilisation of a segment of the coiled coil could possibly allow this rotation [29]. Another possibility is that H2 rotates after its interaction with the microtubule (see text). Stage 3: H2 attaches to the protofilament, releases ADP and undergoes a major conformational change, probably stimulating the $ADPP_i$ to ADP transition in H1 and its subsequent detachment. Stage 4: H1 swings past H2 to complete one step. The relative positions of the two heads are now as in stage 1.

state, shows that to achieve this type of movement the dimer must rotate at some stage during the cycle. It is interesting to compare our observations with the usual schematic representations of the stepping cycle shown in the literature, in which the two motor domains are usually represented as standing upright on a protofilament. A step occurs when the trailing head releases from the protofilament and swings past the attached head whilst rotating into the correct orientation to latch onto the next binding site along the protofilament (in some models there is no rotation). Present indications are that destabilisation of a segment of the coiled-coil region could in principle allow rotation of the free head [29]. In the microtubule–motor complexes that we have observed, the attached motor domain lies parallel to, and stretches full-length along a protofilament. Because we never see two attached heads or a trailing free head, the implication is that these are transient states not easily mimicked in our static experiments. Such states probably occupy only a short time compared to the total ATP hydrolysis cycles of the two heads. In addition, according to our results, once the leading free head attaches to the next site along the protofilament it needs to rotate through ~180° to bring the dimer back to its original orientation one step further along the protofilament. This raises considerable conceptual problems, and at the present time we can only speculate as to how this might be achieved: rotation of the free head could occur before attachment provided that the coiled coil can unzip appropriately; an ~180° rotation could take place after the free head makes an initial contact, which induces release of the trailing head, and so forth.

We also need to take account of biochemical and kinetic evidence that kinesin–ADP has a low affinity for microtubules whereas kinesin–ATP, kinesin–$ADPP_i$ and kinesin without nucleotide all have a much stronger affinity [15]. Moreover, in the presence of microtubules the kinesin ATPase rate is increased ~1000-fold probably by accelerating ADP release, which is the rate limiting step in the cycle [16,27,30]. Recently, it has been shown that each 8 nm step involves one ATP hydrolysis event [31,32] and that the direction of movement is not controlled solely by the catalytic domain [33,34]. Figure 2a shows a scheme in which the ATP hydrolysis cycles of the two heads could be coordinated with their attached and unattached states as shown in Figure 2b.

Biological implications

Kinesins are motor proteins that hydrolyse adenosine triphosphate to generate movement along microtubules.

This movement is required for a variety of intracellular processes, including mitosis, transport between organelles, and the movement of vesicles along the axon. To understand these processes, it is important to determine how the motor proteins interact with and move along microtubules. We have investigated the structural aspects of these questions by determining the three-dimensional structure of the kinesin dimer (a processive microtubule motor) complexed with microtubules in the presence of apyrase and the three nucleotides 5′-adenylylimidodiphosphate (AMP–PNP), ADP–AlF_4 and ADP. The nucleotides were used to mimic the four essential phases of the kinesin ATPase cycle: kinesin without nucleotide, kinesin–ATP, kinesin–$ADPP_i$, and kinesin–ADP. In all nucleotide states the kinesin dimer attaches to a microtubule using one motor domain; the other motor domain hangs free. Only the free domain changes conformation in the presence of different nucleotides, suggesting that it, or the region connecting it to the attached domain, is the determinant of directionality. The free domain also has a high degree of flexibility, suggesting that the search for the next attachment site along a protofilament could be a thermally activated process. Overall, these results provide the first structural information on the behaviour of the kinesin dimer interacting with microtubules in the presence of different nucleotides, thereby extending the data available for modelling the process by which these molecular motors move along microtubules.

Materials and methods

Protein preparation and purification

Tubulin was purified as described previously [6] and stored in 50 ml aliquots at −80°C. Microtubules were assembled at 1 mg/ml for 1 h at 37°C in assembly buffer (100 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, ~50 μM GTP and 10 μM taxotere, pH 6.8 with NaOH). Long stable microtubules were obtained after 2 or 3 days at room temperature. To eliminate free GTP or GDP, they were centrifuged at 12000 rpm for 15 min, the pellet was gently resuspended in GTP-free assembly buffer. Recombinant *Drosophila* kinesin (DKH392, amino acids 1–392) was expressed and purified as described [16].

Specimen preparation and electron microscopy

Microtubules were diluted tenfold with 6 μM DKH392 in phosphate buffer (10 mM sodium phosphate, 50 mM NaCl, 1 mM $MgCl_2$ and 1 mM EGTA, pH 7.4) and incubated for 5 min with either 1 mM AMP–PNP, 1–2 mM ADP, 1 mM ADP–AlF_4 or in the presence of apyrase (25 mU at 30°C). 4 ml samples were applied to holey carbon grids, briefly blotted and rapidly plunged into liquid ethane cooled to liquid nitrogen temperature. Specimens were observed at 200 keV in a Phillips CM 200 using a Gatan 626 cryoholder. Images were recorded on Kodak S.O 163 film at 27500 × magnification and a 2–3 μm defocus. Micrographs were digitalized with an Optronics P1000 at a sampling raster of 12.5 μm and then transferred to Silicon Graphics workstations.

Three-dimensional reconstruction

As described previously [6], reconstructions were carried out and visualised by standard helical reconstruction methods using SUPRIM and SYNUVIEW software, in house and MRC routines. Final maps were generated from the amplitudes and phases of seven layer lines extending to ~35 Å resolution in the computed diffraction pattern. Amplitudes and phases of each layer line are similar in the three states studied except for the $n = -2$, $n = 13$ and $n = -17$ layer lines grouped at a spacing close to 80 $Å^{-1}$. These differences are mainly due to the variations observed in the second head. Each reconstruction represents an average of 10–15 data sets (near or far side), selected after comparison with a reference on the basis of their phase residual (< 35°). No corrections were made for the contrast transfer function and the resolution is ~35 Å. To visualize the maps, isodensity surfaces were calculated for a protein density of 1.4 g/cm^3.

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